



TITLE:

A new species of *Orobdella* (Hirudinida: Arhynchobdellida: *Orobdellidae*) from Primorye Territory, Russian Far East

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1 **A new species of *Orobdella* (Hirudinida: Arhynchobdellida: Orobdellidae) from**
2 **Primorye Territory, Russian Far East**

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14
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20

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22

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29

30 **Geolocation information**

31

32 KUZ Z2068, Z2070 (point): 43.129645°N, 132.798943°E

33 KUZ Z2069 (point): 43.129113°N, 132.798925°E

34

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ABSTRACT

A quadrannulate leech species, *Orobdella ghilarovi* sp. nov., from Primorye Territory in the southern Russian Far East is described. Phylogenetic analyses using nuclear 18S rRNA, 28S rRNA, histone H3, mitochondrial cytochrome *c* oxidase subunit I, tRNA^{Cys}, tRNA^{Met}, 12S rRNA, tRNA^{Val}, 16S rRNA, tRNA^{Leu} and NADH dehydrogenase subunit 1 markers showed that *O. ghilarovi* formed a fully supported clade with a monophyletic lineage comprising two species: *O. kawakatsuorum* and *O. koikei*, inhabiting Hokkaido, Japan. Quadrannulate *Orobdella* leeches distributed in the southern Primorye Territory have been identified as *O. whitmani*, which was originally described in Japan; these past records should be amended based on the taxonomic conclusions of this study.

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KEYWORDS

Erpobdelliformes; terrestrial; macrophagous; molecular phylogeny; misidentification

Introduction

57 The genus *Orobdella* Oka, 1895 is an erpobdelliform leech taxon of the terrestrial
58 macrophagous species inhabiting Far East Asia (Sawyer 1986; Ocegüera-Figueroa et al. 2011;
59 Nakano et al. 2012). *Orobdella* leeches are characterised by their mid-body somite annulation
60 that can be grouped into three types, i.e. 4- (quadr-), 6- (sex-), and 8- (oct-) annulate, and their
61 possession of the gastroporal duct in their digestive tract that receives a spermatophore during
62 copulation (Nakano 2017a, 2017b). They can be also grouped into three morpho-types
63 according to the body length of mature individuals, i.e. small, middle, and large-types
64 (Nakano 2017a).

65 This genus now consists of 20 species: most of them, i.e. 17 species, are distributed in the
66 Japanese Archipelago (Nakano 2017a, 2017b, 2018); one species was recorded in the Korean
67 Peninsula and adjacent islets including Tsushima Island, and two species were described in
68 Taiwan (Nakano and Lai 2012, 2017). Until the 1960s, however, the genus *Orobdella*
69 comprised only three species described in Japan: the quadrannulate *O. whitmani* Oka, 1895,
70 the sexannulate *O. ijimai* Oka, 1895, and the octannulate *O. octonaria* Oka, 1895. In the late
71 1960s, quadrannulate *Orobdella* individuals were recorded around Vladivostok City in
72 Primorye Territory, Russian Far East, and they were identified as *O. whitmani* (Ghilarov et al.
73 1969). Although their identification in the Russian Far East *Orobdella* was followed by
74 subsequent works (Ghilarov and Perel 1971; Lukin 1976; Kurcheva 1977), it was also

75 suggested that the Russian quadrannulate *Orobdella* might be an undescribed species rather
76 than *O. whitmani* (Nakano 2012).

77 Quadrannulate *Orobdella* leeches were newly collected from near Vladivostok. In line
78 with the results of the morphological examination and molecular phylogenetic analyses of the
79 newly obtained specimens, they are described as a new species herein.

80

81 **Material and methods**

82 ***Sampling and morphological examination***

83 Leeches were collected from a locality in Anisimovka, Primorye Territory, southern Russian
84 Far East. When possible, elevation and geographical coordinates for the collection site were
85 obtained using a Garmin eTrex[®] GPS unit.

86 Specimens were relaxed by the gradual addition of absolute ethanol (EtOH) to
87 freshwater, and then fixed in absolute EtOH. For DNA extraction, botryoidal tissue was
88 removed from the posterior part around the caudal sucker of every specimen, and then
89 preserved in absolute EtOH. The remainder of the body was fixed in 10% formalin and
90 preserved in 70% EtOH. Four measurements were taken: body length (BL) from the anterior
91 margin of the oral sucker to the posterior margin of the caudal sucker, maximum body width
92 (BW), caudal sucker length (CL) from the anterior to the posterior margin of the sucker, and
93 caudal sucker width (CW) from the right to the left margin of the sucker. Examination,

dissection, and drawing of the specimens were conducted using a stereoscopic microscope with a drawing tube (Leica M125; Leica Microsystems, Wetzlar, Germany). Specimens used in this study were deposited in the Zoological Collection of Kyoto University (KUZ).

The numbering convention is based on Moore (1927): body somites are denoted by Roman numerals, and the annuli in each somite are given alphanumeric designations.

Molecular phylogenetic analyses

The phylogenetic position of the new *Orobdella* species within the genus was determined based on three nuclear and three mitochondrial markers: 1) 18S rRNA, 2) 28S rRNA, 3) histone H3, 4) cytochrome *c* oxidase subunit I (COI), 5) tRNA^{Cys}, tRNA^{Met}, 12S rRNA, tRNA^{Val} and 16S rRNA (tRNA^{Cys}–16S), and 6) tRNA^{Leu} and NADH dehydrogenase subunit 1 (tRNA^{Leu}–ND1). Methods for the genomic DNA extraction, PCR and cycle sequencing reactions were elucidated in Nakano and Lai (2016, 2017). In total, 14 sequences were newly obtained in this study and deposited with the International Nucleotide Sequence Database Collaboration (INSDC) through DNA Data Bank of Japan (Table 1).

According to the previous phylogenetic study (Nakano et al. 2018), eight OTUs were selected as the outgroup for the present phylogenetic analyses (Table 1). The alignments of H3 and COI were trivial, as no indels were observed. The sequences of the other markers were aligned using MAFFT v. 7.407 L-INS-i (Katoh and Standley 2013). The lengths of the

113 18S, 28S, H3, COI, tRNA^{Cys}-16S, and tRNA^{Leu}-ND1 sequences were 1844, 2823, 328, 1267,
114 1182, and 640 bp, respectively. The concatenated sequences yielded 8084 bp of aligned
115 positions.

116 Phylogenetic trees were inferred using maximum likelihood (ML) and Bayesian inference
117 (BI). The ML phylogeny was inferred using RAxML v. 8.2.8 (Stamatakis 2014) with the
118 substitution model set as GTRCAT, immediately after nonparametric bootstrapping (BS) was
119 conducted with 1000 replicates. The best-fit partition scheme was identified with the
120 Bayesian information criterion using PartitionFinder v. 2.1.1. (Lanfear et al. 2017) with the
121 “greedy” algorithm (Lanfear et al. 2012): 18S, plus H3 1st and 2nd positions/28S/H3 3rd
122 position/COI 1st position/each COI and ND1 2nd position/each COI and ND1 3rd
123 position/tRNA^{Cys}, tRNA^{Met}, 12S and tRNA^{Val}, tRNA^{Leu} and ND1 1st position/16S. BI and
124 Bayesian posterior probabilities (PPs) were estimated using MrBayes v. 3.2.6 (Ronquist et al.
125 2012). The best-fit partition scheme and models for each partition were selected with the
126 Bayesian information criterion using PartitionFinder with the “greedy” algorithm: for 18S and
127 H3 1st position, K80+I+G; GTR+I+G for 28S; JC+I for H3 2nd position; HKY+G for H3 3rd
128 position; GTR+G for COI 1st position; GTR+I+G for 2nd positions of COI and ND1;
129 HKY+I+G for 3rd positions of COI and ND1; GTR+I+G for tRNA^{Cys}-tRNA^{Val} and ND1 1st
130 position; and GTR+G for 16S. Two independent runs of four Markov chains were conducted
131 for 20 million generations, and the tree was sampled every 100 generations. The parameter

estimates and convergence were checked using Tracer v. 1.7.1 (Rambaut et al. 2018), and the first 50001 trees were discarded based on the results.

Pairwise comparisons of uncorrected p -distances for respective COI (1267 bp), tRNA^{Cys}–16S (1032 bp of aligned positions), and tRNA^{Leu}–ND1 (630 bp) sequences obtained from the newly identified *Orobdella* were calculated using MEGA7.0.16 (Kumar et al. 2016). Prior to the calculation, tRNA^{Cys}–16S sequences were aligned using MAFFT FFT-NS-2.

Taxonomy

Genus *Orobdella* Oka, 1895

Orobdella ghilarovi sp. nov.

(Figures 1–3)

Orobdella whitmani: Ghilarov et al., 1969: 235–236, fig. 1; Ghilarov and Perel, 1971: table 1; Lukin, 1976: 464–466, figs. 288, 289.

Type material

Holotype. KUZ Z2068 (Figure 1), dissected, collected from under a stone along a forest road, Anisimovka (43.129645°N, 132.798943°E; elev. 435 m), Shkotovsky District, Primorye

151 Territory, Russia, by Takafumi Nakano, on 21 August 2018.

152

153 ***Paratypes.*** In total 2 individuals collected from around the type locality on 21 August 2018:

154 KUZ Z2069 (43.129113°N, 132.798925°E; elev. 435 m) by Takafumi Nakano, and KUZ

155 Z2070 (43.129645°N, 132.798943°E; elev. 435 m) by Larisa Prozorova.

156

157 ***Diagnosis***

158 Body length of mature individual exceeding 60 mm (67.2 mm in maximum length). Somite

159 IV uniannulate, somites VIII–XXV quadrannulate. Clitellum in somite XI b5 to somite XIII

160 a2. Male gonopore in middle of somite XI b6, female gonopore in middle of somite XIII a1,

161 behind gastropore, gonopores separated by $1/2 + 4 + 1/2$ annuli. Pharynx reaching to somite

162 XIII/XIV. Gastropore conspicuous, in middle of somite XIII a1. Gastroporal duct bulbous.

163 Paired epididymides in somites XVI–XIX, occupying 10 annuli. Atrial cornua developed,

164 ovate.

165

166 ***Description***

167 Body firm and muscular, elongate, with constant width in caudal direction, dorsoventrally

168 compressed, BL 67.2 mm, BW 5.2 mm (Figure 1(a), 1(b)). Caudal sucker ventral, elliptic, CL

169 2.8 mm, CW 3.2 mm (Figures 1(b), 2(d)).

170 Somite I completely merged with prostomium (Figure 2(a)). Somites II (= peristomium),
171 III and IV uniannulate (Figure 2(a)). Somite V biannulate, ($a_1 + a_2$) = a_3 ; a_3 forming
172 posterior margin of oral sucker (Figure 2(a), 2(b)). Somites VI and VII triannulate, $a_1 = a_2 =$
173 a_3 (Figure 2(a), 2(b)). Somites VIII–XXV quadrannulate, $a_1 = a_2 = b_5 = b_6$ (Figure 2(a–e)).
174 Somite XXVI triannulate, $a_1 > a_2 < a_3$ ($b_5 = b_6$ dorsally); a_3 being ventrally last complete
175 annulus (Figure 2(c), 2(d)). Somite XXVII uniannulate with slight dorsolateral furrow on
176 respective sides (Figure 2(c)). Anus behind somite XXVII; post-anal annulus absent (Figure
177 2(c)).

178 Somite X b_5 and somite XIII a_2 , respectively, being first and last annuli of clitellum
179 (Figure 2(e)).

180 Male gonopore in middle of somite XI b_6 (Figure 2(e)). Female gonopore in middle of
181 somite XIII a_1 , inconspicuous, located posterior to gastropore (Figure 2(e), 2(f)). Gonopores
182 separated by $1/2 + 4 + 1/2$ annuli (Figure 2(e)).

183 Anterior ganglionic mass in somite VI a_2, a_3 and somite VII a_1 . Ganglion VII in a_2 .
184 Ganglion VIII in a_2 and b_5 . Ganglia IX–XII, of each somite, in a_2 (Figure 3(c)). Ganglion
185 XIII in a_2 and b_5 (Figure 3(c)). Ganglia XIV–XXIV, of each somite, in a_2 (Figure 3(c)).
186 Ganglion XXV in a_1 . Ganglion XXVI in somite XXV b_6 . Posterior ganglionic mass in somite
187 XXVI a_1 and a_2 .

188 Eyes in 3 pairs, 1st pair dorsally on somite II/III, 2nd and 3rd pairs dorsolaterally on

189 posterior margin of somite V (a1 + a2) (Figure 2(a)). Papillae numerous, minute, hardly
190 visible, one row on every annulus.

191 Nephridiopores in 17 pairs, each situated ventrally at posterior margin of a1 of each
192 somite in somites VIII–XXIV (Figure 2(b), 2(d), 2(e)).

193 Pharynx agnathous, euthylaematous, reaching to somite XIII/XIV (Figure 3(a)). Crop
194 tubular, acaecate, reaching to somite XIX b5/b6 (Figure 3(b)). Intestine tubular, reaching to
195 somite XXIII b5, with 1 pair of pouch-shaped intestinal caeca in somite XIX a2–b6 (Figure
196 3(b)); each caecum opening in behind junction between crop and intestine in somite XIX b6,
197 then ascending to somite XIX a2. Rectum tubular, thin-walled, straight. Gastropore
198 conspicuous, ventral, on middle of somite XIII a1 (Figure 2(e), 2(f)). Gastroporal duct
199 bulbous, winding at junction with gastropore, reaching to somite XIII b6 (Figure 3(a)).

200 Testisacs multiple; on right side, in somite XIX b5 to smite XXV a2, in total ~28
201 testisacs, 1 in XIX, 5 in XX, 4 in XXI, 6 in XXII, 5 in XXIII, 5 in XXIV, 2 in XXV; on left
202 side, in somite XIX b6 to somite XXV b5, in total ~30 testisacs, 1 in XIX, 5 in XX, 5 in XXI,
203 6 in XXII, 5 in XXIII, 5 in XXIV, 3 in XXV. Paired epididymides in somite XVI b6 to somite
204 XIX a1, occupying 10 annuli (Figure 3(c)). Paired ejaculatory duct in somite XI b5 to somite
205 XVI b6 (Figure 3(c)); coiled in position posterior to ovisacs; each duct crossing ventrally
206 beneath each ovisac, then nearly straight in position anterior to ovisacs; each widening from
207 respective junction with epididymis, narrowing at junction with atrial cornua, then turning

208 proximally toward atrial cornua without pre-atrial loop (Figure 3(c–f)). Pair of muscular atrial
209 cornua developed, ovate, in somite XI b5 and b6 (Figure 3(c–f)). Atrium short, muscular,
210 globular in somite XI b5 and b6 (Figure 3(d–f)). Penis sheath and penis absent.
211 Paired ovisacs globular, in somite XIII a1–b5 (Figure 3(c), 3(g)). Oviduct thin-walled,
212 left oviduct crossing ventrally beneath nerve cord (Figure 3(c), 3(g)); both oviduct converging
213 into common oviduct in somite XIII a1/a2. Common oviduct thin-walled, short, directly
214 descending to female gonopore (Figure 3(g)).

215

216 ***Variation***

217 Measurements ($n = 2$, paratypes only): BL 35.0–36.1 mm, BW 3.1 mm, CL 1.5–1.6 mm, CW
218 1.9–2.1 mm. Somite XXVI biannulate, $a1 < (a2 + a3)$, $(a2 + a3)$ with slight dorsolateral
219 furrow on respective sides, or triannulate $a1 > a2 = a3$. Small unpaired eye dorsally on right
220 posterior margin of somite IV (KUZ Z2069).

221

222 ***Colouration***

223 In life, dorsal surface yellow (Figure 1(c)), or yellowish brown; ventral surface whitish
224 yellow, or greyish white; clitellum, when obvious, paler than other body parts (Figure 1(c)).
225 Colour faded in preservative; dark mid-dorsal line present from somite VIII a1 to somite
226 XXVII in holotype (Figure 1(a)).

227

228 ***Distribution***229 According to the previous records of quadrannulate *Orobdella* leeches from around230 Vladivostok (Ghilarov et al. 1969; Ghilarov and Perel 1971), *O. ghilarovi* inhabits the Ussuri

231 Nature Reserve in addition to the type locality.

232

233 ***Natural history***

234 This species was found curled up under rocks in moist habitats along a forest road. Since a

235 mature individual (= holotype) bearing a slight clitellum and developed testisacs as well as

236 ovisacs was collected on 21 August 2018, the reproductive season of this species may be

237 around August.

238

239 ***Etymology***

240 The specific name is a noun in the genitive case formed directly from the name of the late Dr

241 Merkuriy Sergeevich Ghilarov, who provided the first record of *Orobdella* leeches from

242 Primorye Territory (Ghilarov et al. 1969).

243

244 ***Molecular analyses results***245 The BI tree (mean $\ln L = -45227.02$; Figure 4) for determining the phylogenetic position of

246 *O. ghilarovi* had an almost identical topology to that of the ML tree ($\ln L = -46910.15$; not
247 shown). *Orobodella ghilarovi* was a sister taxon of a well-supported clade (BS = 100%, PP =
248 1.0) including the two species, *O. kawakatsuorum* Richardson, 1975 and *O. koikei* Nakano,
249 2012, inhabiting Hokkaido, northern Japan, and adjacent islets (Nakano 2012; Nakano and
250 Gongalsky 2014). The monophyly of the continental *O. ghilarovi* + Hokkaido clade was fully
251 supported (BS = 100%, PP = 1.0). This clade was a sister lineage to a monophyletic group
252 (BS = 100%, PP = 1.0) consisting of the other 18 species known from the Korean Peninsula,
253 Taiwan, and the other Japanese islands in the south of Hokkaido. This major monophyletic
254 group was divided into two sub-lineages: a lineage corresponded to *O. tsushimensis* Nakano,
255 2011 that is distributed in the Korean Peninsula and adjacent islands; and another clade (BS =
256 77%, PP = 0.97) contained the remaining 17 species.

257 The respective COI (LC431609–LC431610), and tRNA^{Leu}–ND1 (LC431613–LC431615)
258 sequences, which were obtained from the holotype and two paratypes of *O. ghilarovi*, did not
259 exhibit any variations among them. Although base-compositions of the tRNA^{Cys}–tRNA^{Val}
260 regions in the three tRNA^{Cys}–16S sequences (LC431616–LC431618) dovetailed completely
261 with each other, the remaining 16S sequences were slightly variable, and thus the pairwise
262 tRNA^{Cys}–16S uncorrected *p*-distance within three individuals of *O. ghilarovi* was 0.20%.

263

264 **Remarks**

265 *Orobdella ghilarovi* clearly belongs to *Orobdella* as it possesses the generic diagnostic
266 features defined by Nakano (2016a). Its molecular phylogenetic position also supports the
267 present genus-level affinity of this new species. Additionally, the mitochondrial DNA
268 sequences obtained from the present three specimens fully supported their taxonomic identity
269 belonging to the same species. Although only one mature individual of the new species (=
270 holotype) could be obtained, the dissected holotype provided sufficient morphological
271 features of the new species along with the two undissected paratypes.

272 According to previous studies (Nakano 2010, 2011, 2012; Nakano and Lai 2012; Nakano
273 2014; Nakano and Seo 2014; Nakano 2016a, 2016b, 2017b; Nakano and Lai 2017; Nakano
274 2018), therefore, the new species can be distinguished from the 12 quadrannulate congeners,
275 i.e. *O. angustata* Nakano, 2018, *O. brachyepididymis* Nakano, 2016a, *O. esulcata* Nakano,
276 2010, *O. kanaekoikeae* Nakano, 2017b, *O. kawakatsuorum*, *O. koikei*, *O. masaakikuroi*wai
277 Nakano, 2014, *O. meisai* Nakano and Lai, 2017, *O. naraharaetmagarum* Nakano, 2016b, *O.*
278 *tsushimensis* and *O. whitmani*, by the following combination of characteristics (Table 2):
279 middle-type body length, uniannulate somite IV, quadrannulate somite XXV, $1/2 + 4 + 1/2$
280 annuli between gonopores, pharynx reaching to posterior XIII, bulbous gastroporal duct,
281 epididymides in somites XVI–XIX that occupy 10 annuli, and developed ovate atrial cornua.
282 *Orobdella ghilarovi* is clearly distinguishable from the six sexannulate and two octannulate
283 species by its mid-body somites that are quadrannulate.

284 Since internal characteristics of the Russian quadrannulate *Orobdella* leeches, which
285 were reported as *O. whitmani*, were not provided by Ghilarov et al. (1969), their precise
286 taxonomic account remains unclarified. However, it was stated that their male and female
287 gonopores opened in the middle of the respective annuli, and the individuals possessed 4
288 complete annuli between the gonopores (Ghilarov et al. 1969), i.e. they bore $1/2 + 4 + 1/2$
289 annuli between the gonopores. This characteristic is coincident with the feature of *O.*
290 *ghilarovi*, and the Ghilarov's specimens were collected from a forest near Kangauz Settlement
291 (Ghilarov et al. 1969), which is the old Chinese name of the type locality of the new species,
292 Anisimovka Settlement renamed in 1972. Accordingly, the *Orobdella* leeches reported by
293 Ghilarov et al. (1969) and Ghilarov and Perel (1971) are judged herein as *O. ghilarovi*.
294 Additionally, *Orobdella* individuals were also collected from two other locations of Primorye
295 Territory, which are more distant from Vladivostok, i.e. in the Khasan Settlement, the vicinity
296 near Russian/North Korean state boundary, and nearby the Chuguevka Settlement (Kurcheva
297 1977). Their taxonomic account should be revised by future taxonomic studies.

298 The obtained molecular phylogenies revealed that *O. ghilarovi* is genetically close to *O.*
299 *kawakatsuorum* and *O. koikei* inhabiting Hokkaido and adjacent islets; the new species did
300 not form a monophyletic group with *O. tsushimensis*, which is distributed in the continental
301 Korean Peninsula and adjacent islets (Nakano 2011; Nakano and Seo 2012, 2014). This result
302 highlighted the close relationships among the three species, i.e. *O. ghilarovi*, *O.*

303 *kawakatsuorum* and *O. koikei*, with insight into the biogeographical history of the genus

304 *Orobdella*. Future faunal and systematic studies of *Orobdella* leeches in the Russian Far East

305 including Sakhalin Island will lead us to a better understanding of the evolutionary and

306 biogeographical histories of this terrestrial macrophagous leech group. Moreover, a recent

307 molecular phylogenetic study shed light onto a distinctive phylogenetic status of the

308 American terrestrial macrophagous species *Americobdella valdiviana* (Philippi, 1872), and

309 split Arhynchobdellida Blanchard, 1894 into the three suborders (Tessler et al. 2018), i.e.

310 Americobdelliformes Siddall, de Carle and Tessler in Tessler et al., 2018, Erpobdelliformes

311 Sawyer, 1986 and Hirudiniformes Caballero, 1953. Since the genus *Orobdella* forms a basal

312 phylogroup within Erpobdelliformes (Nakano et al. 2012, 2018), future evolutionary studies

313 that focus on phylogenetic positions of this genus and the American terrestrial macrophagous

314 taxa will elucidate a key evolutionary event of the arhynchobdellidan leeches.

315

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411 **Table 1.** Samples used for phylogenetic analyses; the information on the vouchers is accompanied by the INSDC accession numbers

Species	Voucher ¹	INSDC# ²					
		18S	28S	Histone H3	COI	tRNA ^{Cys} –16S	tRNA ^{Leu} –ND1
<i>Orobodella</i>							
<i>O. ghilarovi</i> sp. nov.	KUZ Z2068 Holotype	LC431608*	LC431607*	LC431612*	LC431609*	LC431616*	LC431613*
<i>O. ghilarovi</i> sp. nov.	KUZ Z2069 Paratype				LC431610*	LC431617*	LC431614*
<i>O. ghilarovi</i> sp. nov.	KUZ Z2070 Paratype				LC431611*	LC431618*	LC431615*
<i>O. angustata</i>	KUZ Z1439 Holotype	LC323140	LC431606*	LC323138	LC323139	LC323141	LC323137
<i>O. brachyepididymis</i>	KUZ Z1673 Holotype	LC106319	LC274535	LC106321	LC106320	LC106318	LC106322
<i>O. dolichopharynx</i>	KUZ Z120 Holotype	AB663665	LC274541	AB698876	AB679680	AB679681	AB828558
<i>O. esulcata</i>	KUZ Z29 Holotype	AB663655	LC274538	AB698873	AB679664	AB679665	AB828555
<i>O. ijimai</i>	KUZ Z110 Topotype	AB663659	LC274542	AB698877	AB679672	AB679673	AB828559
<i>O. kanaekoikeae</i>	KUZ Z1747 Holotype	LC184551	LC274533	LC184553	LC184552	LC184550	LC184554
<i>O. kawakatsuorum</i>	KUZ Z167 Topotype	AB663661	LC274544	AB698878	AB679704	AB679705	AB828561
<i>O. ketagalan</i>	KUZ Z208 Holotype	AB704785	LC274546	AB704786	AB704787	AB828582	AB828563
<i>O. koikei</i>	KUZ Z156 Holotype	AB698883	LC274543	AB698882	AB679688	AB679689	AB828560
<i>O. masaakikuroiwai</i>	KUZ Z694 Holotype	AB938003	LC274530	AB938013	AB938006	AB937997	AB938016
<i>O. meisai</i>	KUZ Z1917 Holotype	LC314423	LC431605*	LC314425	LC314424	LC314422	LC314426
<i>O. mononoke</i>	KUZ Z224 Holotype	AB698868	LC274547	AB698869	AB698866	AB698867	AB828564
<i>O. nakahamai</i>	KUZ Z1672 Holotype	LC106330	LC274534	LC106332	LC106331	LC106329	LC106333
<i>O. naraharaetmagarum</i>	KUZ Z1652 Holotype	LC087143	LC274531	LC087145	LC087144	LC087142	LC087146
<i>O. octonaria</i>	KUZ Z181 Topotype	AB698870	LC274545	AB698871	AB679708	AB679709	AB828562
<i>O. okanoi</i>	KUZ Z1671 Holotype	LC106341	LC274532	LC106343	LC106342	LC106340	LC106344

<i>O. shimadae</i>	KUZ Z128 Holotype	AB663663	LC274540	AB698875	AB679676	AB679677	AB828557
<i>O. tsushimensis</i>	KUZ Z134 Holotype	AB663653	LC274537	AB698872	AB679662	AB679663	AB828554
<i>O. whitmani</i>	KUZ Z45 Topotype	AB663657	LC274539	AB698874	AB679668	AB679669	AB828556
<i>O. yamaneae</i>	KUZ Z1678 Holotype	LC106349	LC274536	LC106351	LC106350	LC106348	LC106352
Outgroup							
<i>Erpobdella japonica</i>	KUZ Z178	AB663648	LC274512	AB698879	AB679654	AB679655	AB828542
<i>Gastrostomobdella ampunganensis</i>	ZRC.ANN.0083 Holotype	LC274517	LC274516	LC274552	LC274551	LC274564	LC274571
<i>Gastrostomobdella extenta</i>	MUMNH-Hir0001 Holotype	LC274519	LC274518	LC274554	LC274553	LC274565	LC274572
<i>Gastrostomobdella monticola</i>	UNIMAS/A3/BH01/10	AB663649	LC274515	AB698880	AB679656	AB679657	AB828543
<i>Gastrostomobdella</i> cf. <i>monticola</i>	KUHE 56264	LC274514	LC274513	LC274550	LC274549	LC274563	LC274570
<i>Mimobdella japonica</i>	KUZ Z179	AB663650	LC274528	AB698881	AB679658	AB679659	AB828544
<i>Odontobdella blanchardi</i>	KUZ Z180	AB663651	LC274529	AB938012	AB938004	AB937995	AB938014
<i>Salifa motokawai</i>	VNMN 2015.65 Holotype	LC029434	LC274548	LC029435	LC029431	LC029432	LC029433

412 ¹Acronyms: KUHE, Kyoto University, Human and Environmental Studies; KUZ, Zoological Collection of Kyoto University; MUMNH, Mahidol

413 University Museum of Natural History; UNIMAS, Universiti Malaysia Sabah; VNMN, Vietnam National Museum of Nature; ZRC, Zoological

414 Reference Collection, Lee Kong Chian Natural History Museum.

415 ²Sequences marked with an asterisk (*) were obtained for the first time in the present study.

416

417 **Table 2.** Comparisons of morphological characters between *Orobdella ghilarovi* sp. nov. and 12 quadrannulate species

Species	Body length	Somite IV	Somite XXV	Annuli between gonopores	Pharynx length	Gastroporal duct	Epididymides	Atrial cornua
<i>O. ghilarovi</i> sp. nov.	middle	1	4	1/2 + 4 + 1/2	to posterior XIII	bulbous	XVI to XIX	developed, ovate
<i>O. angustata</i>	middle?	1	4	1/2 + 4 + 1/3	to anterior XIV	bulbous	XVIII to XX	developed, hyperbologidal
<i>O. brachyepididymis</i>	small	1	4	1/2 + 4 [+ (< 1/2)]	to anterior XIV	tubular	XX to XXI	small, ovate
<i>O. esulcata</i>	middle	1	4	2/3 + 4 + 1/3	to anterior to posterior XIV	tubular, but bulbous at junction with gastropore	XVI to XX	developed, ovate
<i>O. kanaekoikeae</i>	small	1	4	1/2 + 4 + 1/2	to posterior XIII to anterior XIV	bulbous	XIV to XVIII	developed, ovate
<i>O. kawakatsuorum</i>	middle	2	4	6	to middle to posterior XIV	simple tubular	XVI to XVII	undeveloped
<i>O. ketagalan</i>	middle	1	4	1/2 + 4 + 1/2	to posterior XIV	simple tubular	absent	undeveloped
<i>O. koikei</i>	small	1	3	1/2 + 4 + 1/2	to posterior XIII to anterior XIV	bulbous	XV to XX	developed, ovate
<i>O. masaakikuroiwai</i>	small	1	4	1/2 + 4 + 1/2	to anterior to middle XIV	bulbous	XVI to XVIII	developed, ovate
<i>O. meisai</i>	middle	1	4	5 + 1/4	to posterior XV	rudimentary tubular	absent	absent
<i>O. naraharaetmagarum</i>	small	1	4	1/2 + 4 + 1/2	to posterior XIII	bulbous	XV to XX	developed, ovate
<i>O. tsushimensis</i>	middle	1	4	1/2 + 5	to posterior XIII to posterior XIV	bulbous	XVII to XIX	developed, ovate
<i>O. whitmani</i>	middle	1 or 2	4	1/2 + 4 + 1/2	to anterior to posterior XIV	bulbous	XVI to XVIII	developed, ovate

418

419 Figure captions

420

421 **Figure 1.** *Orobdella ghilarovi* sp. nov., holotype, KUZ Z2068. (a) Dorsal view. (b) Ventral
422 View. (c) Dorsal view of live animal. Scale bars, 5 mm.

423

424 **Figure 2.** *Orobdella ghilarovi* sp. nov., holotype, KUZ Z2068. (a) Dorsal view of somites I–
425 VIII. (b) Ventral view of somites I–VIII. (c) Dorsal view of somites XXIV–XXVII and caudal
426 sucker. (d) Ventral view of somites XXIV–XXVI and caudal sucker. (e) Ventral view of
427 somites X–XIII. (f) Ventral view of gastropore and female gonopore. Abbreviations: af,
428 annular furrow; an, anus; cl, clitellum; fg, female gonopore; gp, gastropore; mg, male
429 gonopore; np, nephridiopore. Scale bars, 2 mm (a–e); 0.2 mm (f).

430

431 **Figure 3.** *Orobdella ghilarovi* sp. nov., holotype, KUZ Z2068. (a) Ventral view of gastroporal
432 duct. (b) Ventral view of junction between crop and intestine with intestinal caeca. (c) Dorsal
433 view of reproductive system including ventral nervous system. (d) Dorsal view of male atrium
434 including position of ganglion XI. (e) Left lateral view of male atrium. (f) Ventral view of
435 male atrium. (g) Dorsal view of female reproductive system including position of ganglion
436 XIII. Abbreviations: ac, atrial cornua; at, atrium; cod, common oviduct; cp, crop; ed,
437 ejaculatory duct; ep, epididymis; gd, gastroporal duct; gp, gastropore; ic, intestinal caecum;

438 in, intestine; od, oviduct; ov, ovisac; ph, pharynx. Scale bars, 2 mm (a, b); 5 mm (c); 0.5 mm

439 (d–g).

440

441 **Figure 4.** Bayesian inference tree for 8084 bp of nuclear 18S rRNA, 28S rRNA and histone

442 H3 plus mitochondrial COI, tRNA^{Cys}, tRNA^{Met}, 12S rRNA, tRNA^{Val}, 16S rRNA, tRNA^{Leu} and

443 ND1 markers. Inset shows real branch lengths. Numbers on nodes represent bootstrap values

444 for maximum likelihood and Bayesian posterior probabilities.

445

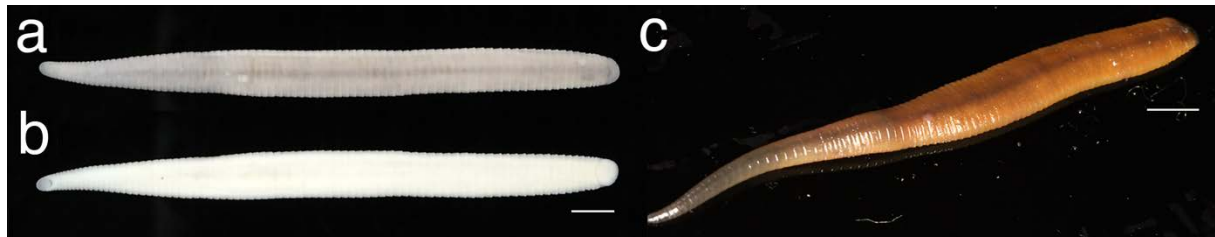


Figure 1. *Orobdella ghilarovi* sp. nov., holotype, KUZ Z2068. (a) Dorsal view. (b) Ventral View. (c) Dorsal view of live animal. Scale bars, 5 mm.

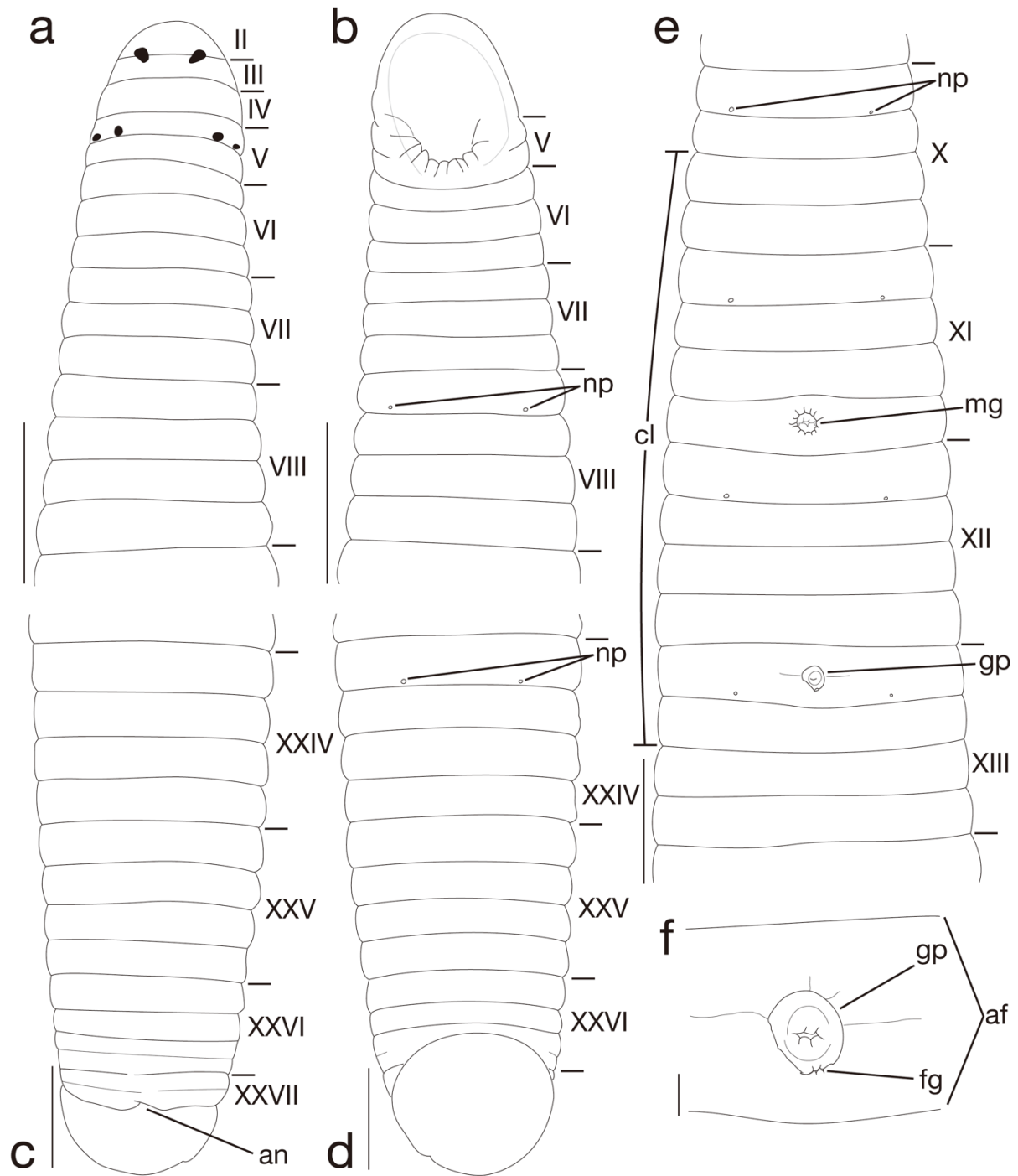


Figure 2. *Orobdella ghilarovi* sp. nov., holotype, KUZ Z2068. (a) Dorsal view of somites I–VIII. (b) Ventral view of somites I–VIII. (c) Dorsal view of somites XXIV–XXVII and caudal sucker. (d) Ventral view of somites XXIV–XXVI and caudal sucker. (e) Ventral view of somites X–XIII. (f) Ventral view of gastropore and female gonopore. Abbreviations: af, annular furrow; an, anus; cl, clitellum; fg, female gonopore; gp, gastropore; mg, male gonopore; np, nephridiopore. Scale bars, 2 mm (a–e); 0.2 mm (f).

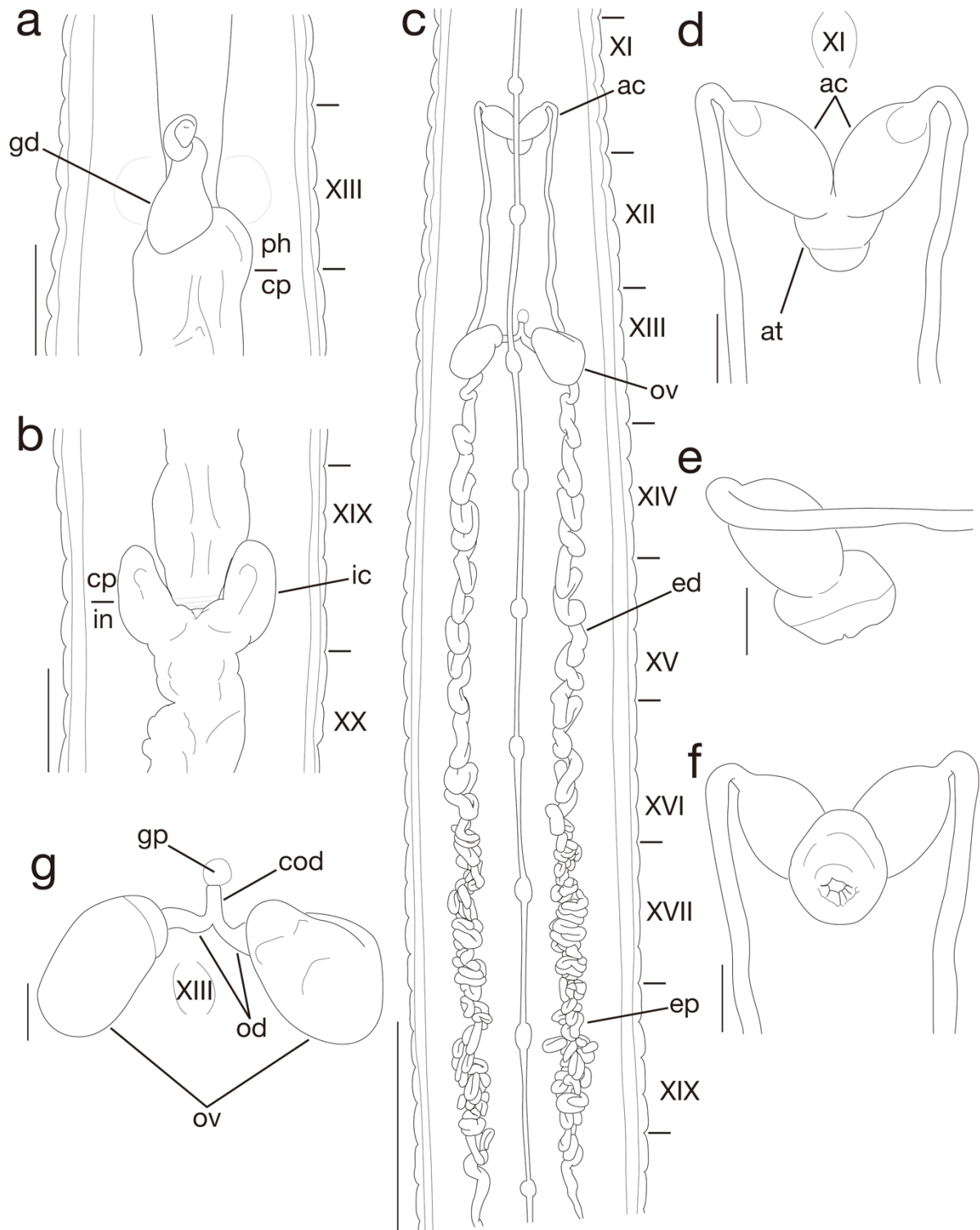


Figure 3. *Orobdella ghilarovi* sp. nov., holotype, KUZ Z2068. (a) Ventral view of gastroporal duct. (b) Ventral view of junction between crop and intestine with intestinal caeca. (c) Dorsal view of reproductive system including ventral nervous system. (d) Dorsal view of male atrium including position of ganglion XI. (e) Left lateral view of male atrium. (f) Ventral view of male atrium. (g) Dorsal view of female reproductive system including position of ganglion XIII. Abbreviations: ac, atrial cornua; at, atrium; cod, common oviduct; cp, crop; ed, ejaculatory

465 duct; ep, epididymis; gd, gastroporal duct; gp, gastropore; ic, intestinal caecum; in, intestine;
466 od, oviduct; ov, ovisac; ph, pharynx. Scale bars, 2 mm (a, b); 5 mm (c); 0.5 mm (d–g).
467

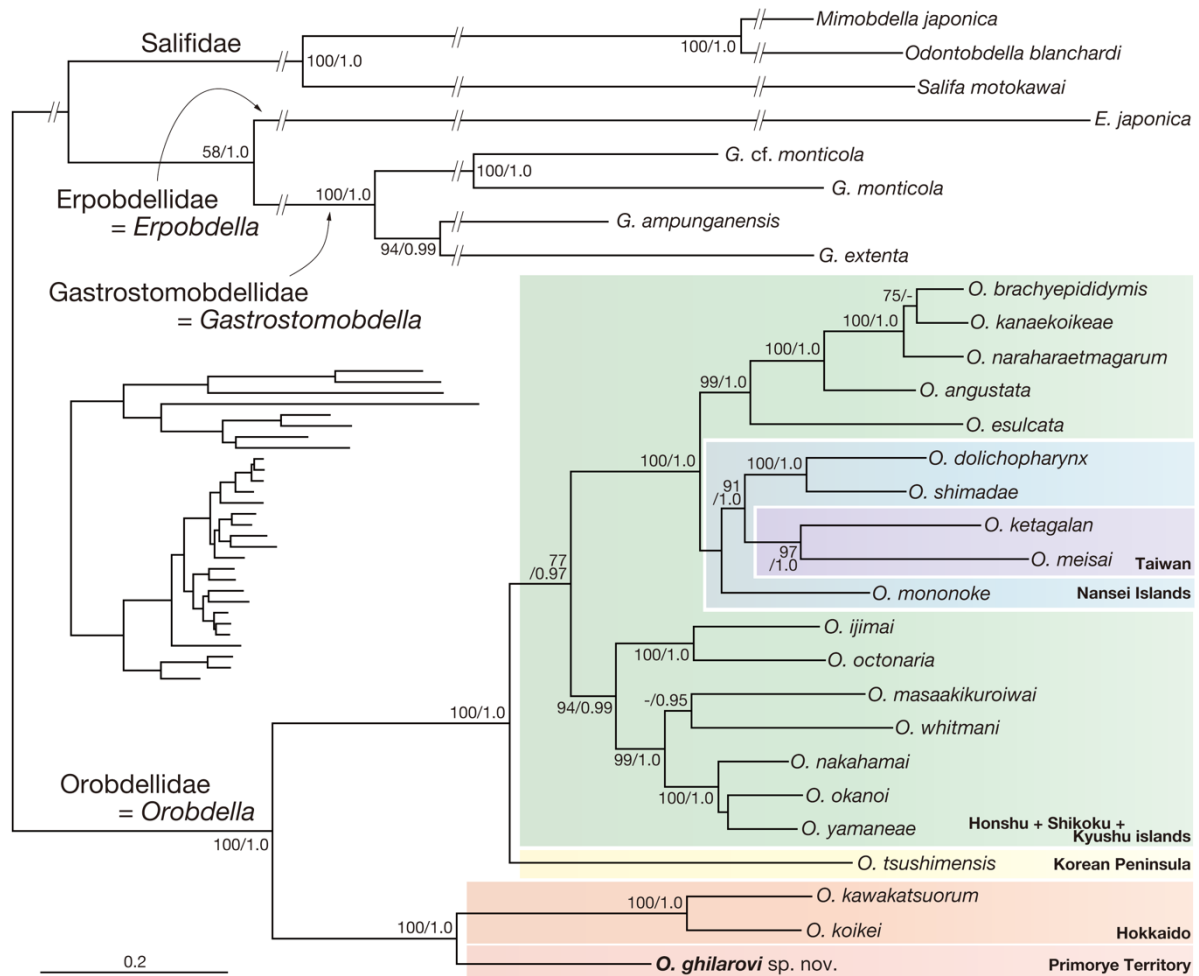


Figure 4. Bayesian inference tree for 8084 bp of nuclear 18S rRNA, 28S rRNA and histone H3 plus mitochondrial COI, tRNA^{Cys}, tRNA^{Met}, 12S rRNA, tRNA^{Val}, 16S rRNA, tRNA^{Leu} and ND1 markers. Inset shows real branch lengths. Numbers on nodes represent bootstrap values for maximum likelihood and Bayesian posterior probabilities.